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PRINCIPAL INVESTIGATOR: Wynne Schiffer, PhD

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, MN 55455

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14. ABSTRACT A major reason for the clinical under treatment of chronic pain in the military lies in the continued, and valid, concern that overtreatment may result in the development of drug dependency, which ultimately results in the need for detoxification and long term treatment of the induced dependency/addiction. Therefore, in an ongoing effort to develop an effective pharmacological approach designed specifically to prevent drug dependence that may result from appropriate therapeutic administration of opiate analgesics such as morphine or prescription medications, our goal is to measure the addictive liability of a standard opiate analgesic (morphine), administered in excess of that required to effectively manage pain in a clinically relevant animal model. We have tested two animal models; the Formalin Paw Test (FPT) and the Acetic Acid (AA) writhing test in the context of a reward model, the Conditioned Place Preference (CPP) test. Our ultimate goal is to determine if γ -vinyl GABA (GVG), a compound that blocks the rewarding effects of a number of addictive drugs but does not itself produce tolerance or dependence can reduce or eliminate the addictive liability of an excessive dose of morphine. This is a novel strategy to eliminate the prevalence of substance abuse in returning and active military personnel, while safely insuring that adequate pain relief will be available without the fear of addiction. In this year of the project, despite construction delays and radiochemistry complications, substantial progress has been made.					
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Introduction

During the latter portion of the last decade, an increase in pain medication prescriptions has promoted a drastic rise in opioid dependence and abuse in both civilian and military populations (1, 2). Prescription drug use has risen especially rapidly in the military population (2). This trend, largely the result of US engagement in two wars over the past decade, has put military personnel and their families at a high risk for drug addiction. The incidence of diagnosed opioid abuse is nearly 7 times higher in the veteran's administration population than in commercial health care plans (3). In fact, Bray and colleagues found that pain medication is the most highly abused of all prescribed drugs in the military (4). Prescription opioid overdose causes more deaths than cocaine and heroine combined (5). Furthermore, prescription opioid abuse represents a growing economic burden on society, a cost estimated at over 55 billion dollars annually (6). Possible drug interventions should be considered and scientific research should prioritize a deeper understanding of the relationship between pain, opiate use and addiction. A prevention option that allows opiates to be administered at levels sufficient to manage pain without the high risk for addiction is essential in order to more successfully care for active duty military personnel and veterans.

One explanation is that opiate abuse begins with an insult that is under treated for pain. An alternate explanation is that doses of opiate analgesics that may be appropriate for initial pain management, ultimately become excessive with the underlying pathology is corrected and the nociceptive input is diminished. In this case, the patient may likely continue to take (require) higher doses of opiate to maintain the desired sense of well being than is necessary to ameliorate the nociceptive stimulus alone.

Through a comprehensive preclinical behavioral-imaging paradigm, our ultimate goal is to demonstrate a strategy in which morphine can be given to military personnel and civilians at doses appropriate for pain relief without the high risk of addiction. By combining multiple behavioral and imaging tools, we expect to show that administration of the drug gamma-vinyl GABA (GVG) prior to morphine treatment effectively eliminates the highly addictive nature of this opiate. In these studies, we will measure addictive liability using Conditioned Place Preference (CPP), assess pain by observing writhing behavior, and track changes in the dopamine reward system with [^{11}C]-raclopride Positron Emission Tomography (PET). Our hypothesis will be tested with these measures that can be meaningfully correlated to draw conclusions about GVG's effect on morphine's addictive properties in the presence of pain.

This proposal wasn't fully transferred to the University of Minnesota until May of 2014, two years after the PI arrived at UMN. Further, construction of the behavioral and radiochemistry laboratories was not completed until 2015. During this period the PI succeeded in obtaining necessary radiation permits, repaired and resolved existing issues with the small animal PET as well as trained a laboratory staff to run and maintain the equipment. Streamlined protocols were developed to incorporate imaging and behavioral measures required for this project. The goal was to ensure that the work of the grant could recommence following completion of the radiochemistry laboratory and training of the radiochemist. A no-cost extension was necessary to re-establish a realistic data collection and reporting period. In the initial months after completion of the radiochemistry facility and training of the radiochemist (4 months for his training), it has been difficult to arrange radiochemical deliveries necessary for time-dependent, longitudinal behavioral studies.

Our original pain model (Formalin Paw Test, FPT) was not conducive to imaging nor was it practical for determining morphine reward in the context of analgesia. However this new knowledge became a key driver for the PI to establish outside collaborations, leading to the development of a solid experimental platform using the Acetic Acid (AA) Writhing test. The final phase of this project will use the AA test combined with Conditioned Place Preference (CPP) and imaging to determine whether GVG effectively blocks the rewarding, but not the analgesic, properties of morphine (Tasks 2a – 2c). **Thus, the limitations of our initial model fostered a new design and a new collaboration that will strengthen both the final and future phases of this project.**

The bulk of recent efforts at the University of Minnesota have been to validate the Task 1 procedures established at the Feinstein Institute and at Brookhaven National Laboratory. Validation of our model is essential for determining the efficacy of our novel pharmacological intervention (Tasks 2a – 2c). **Thus, the major focus of our efforts in the past month that the award has been released has been to set up and validate the Conditioned Place Preference (CPP) and Acetic Acid (AA) protocols in our new laboratory at the University of Minnesota, with the aim of completing Tasks 2a – 2c during this final phase of the project.** The work completed during this reporting period is described below.

1. Optimizing the Place Conditioning Protocol

The supposition that CPP is a reliable and robust marker of addictive liability is central to our approach. We recognize that CPP is neither the only marker for addictive liability nor necessarily infallible since only a positive test can be unambiguously interpreted. Because the behavioral experiments are so critical to the direction of this project, we have placed a major emphasis on combined CPP and AA models together with morphine and GVG treatments in the short time that we have had the award. In the past month, we have successfully established a high-throughput CPP laboratory that can be combined with the AA pain model and tested this with morphine treatment (see below). In this funding period, we have deliberately focused on the translational opportunities of this research to improve pain management in military personnel and all beneficiaries.

At a purely behavioral level in animals, the paradigm of CPP is widely used to model drug-seeking behavior or craving. CPP can be used to reveal the presence of pain and to help in validating mechanisms that can ameliorate it. In our lab, the CPP boxes have three contextually distinct conditioning (also known as pairing) environments that are distinguished by visual and sensory cues, two of which are separated by a middle, neutral environment. Animal subjects typically undergo handling by the experimenter prior to a pre-test phase, where their initial preference for one of the environments is established and the animals are subsequently paired with the rewarding drug or stimulus in the opposite chamber. This type of CPP design assumes that if an animal subject initially prefers one environment, then it will have a preference for that environment regardless of the drug treatment. In other words, the initial pre-test is presumed to be predictive

of the eventual post-treatment test, where animals are given a choice of environments and if they spend more time in the drug-paired environment on the test day, they are thought to be drug seeking or craving. However, in the past month we conducted experiments in which animals were given a series of pre-tests (prior to drug treatment in a specific environment) over 10 days and found that the initial pre-test was not predictive of the last test. Many studies quantitate CPP data by comparing the post-drug test data to the pre-test data, however a random pre-test preference could lead to spurious results when compared to post-drug test data. (see **Figure 1a** for an average of sequential pre-test observations).

There are several major benefits to our choice of the CPP paradigm over our originally proposed Formalin Paw Test:

1. Animal subjects are tested in a drug-free state, 24 hours after their last dose of morphine or AA. This reduces a major confound for simultaneous imaging studies, which would simply be measuring the effects of the drug and not the rewarding properties of the treatment.
2. Experimentally, CPP is amenable to awake animal imaging studies.
3. The AA paradigm can also be performed in standard CPP boxes, and the PI has been to Purdue Pharmaceuticals to learn their technique for administering analogs of Oxycontin to assess attenuation of pain behaviors. Thus, CPP is a sensitive method for measuring the analgesic properties of candidate drugs.
4. Both writhing behavior (the measure of pain in the AA assay) and reward can be measured simultaneously with suspended video tapes, and together with locomotion (see below) an extensive behavioral profile can be derived from each animal subject.
5. CPP is widely accepted by the FDA and commonly used by industry to measure drug reward, lending to the translational relevance of this project and our future goal of extending this strategy to patients.

Although this behavioral technique is widespread, a major methodological controversy exists regarding the issue of pre-conditioned environment preferences (i.e. an animal may have a significant preference for a black environment compared to a white environment prior to any drug pairing).

If experimentally naïve animals naturally spend more time in one environment compared to another, then they are said to possess a pre-existing environment preference and the conditioning apparatus is considered “biased.” As a result of this apparatus bias, proper interpretation of the hypothesized rewarding effects of the treatment is compromised. To anticipate this confound, we sought to design an unbiased apparatus so our animals would not have an initial environment preference.

To test whether our apparatus was biased, drug naïve animals (n=8) were then given free access to the full apparatus for a 5-day period (20-minute sessions, two sessions per day). These sessions are known as “pre-test” sessions, and are commonly used when implementing Place conditioning protocols. The time spent

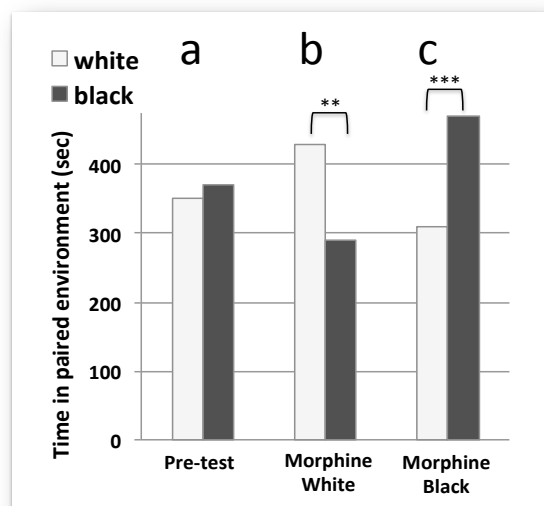


Figure 1. Mean seconds spent in each environment during a 5-day pre-test study (a). It is clear that under the conditions described in the text, our apparatus is not biased and there is no predictive preference for the black or white environment in the absence of drug. Animals were later paired with Morphine (5mg/kg) in a specific environment over an 8-day pairing regimen. Post pairing, animals were tested for place preference. Animals spent more time in their morphine-paired environment on test day (b and c). This data clearly demonstrates that morphine produces a place preference regardless of the conditioning environment. These studies demonstrate that we have set up and validated the conditioning protocol in our lab.

in the black and white environment of the apparatus was recorded using Logitech video software and was scored with dedicated software (TopScan, Reston, VA). Our initial study produced a pre-test preference for the black environment; all animals preferred the black environment compared to the white environment. On average, animals spent 62% of the time in the black environment and 38% of the time the white environment (p-value=0.006) during this 5-day pre-test study (data not shown). In an effort to eliminate this pre-test preference for the black environment, we met with a preclinical behavioral expert and dimmed the lighting in our behavioral lab to 30 lux, then repeated the pre-test study with a second group of animals (n=8).

Dimming the ambient lighting diminished the pre-test preference, and animals spent approximately 52% of the time in the black environment and 48% of the time in the white environment. After we confirmed that our apparatus was unbiased, we successfully conditioned animals (n=8) to a specific environment using morphine (Figure 1b and 1c). A major goal for the upcoming grant period is to investigate whether GVG can block morphine conditioned preference in the presence of pain (Task 2a).

At this point, we will introduce gamma-vinyl GABA (GVG) to our CPP paradigm to determine GVG's ability to prevent the addictive liability of opiate treatment without affecting the pain management properties (Task 2a). The integration of this new behavioral model with imaging promises to make an important preliminary contribution to the health and welfare of military personnel, their families and the general public.

2. Establishing and Validating the Acetic Acid Writhing Protocol

The second major focus of our initial studies has been to set up and validate the Acetic Acid (or Writhing) protocol in our lab. First, the concentration (0.9% – 2% AA) and pretreatment time (0 – 60 minutes) of AA were systematically manipulated, with the goal of identifying the conditions under which AA can reliably produce writhing behavior.

We first examined a range of Acetic Acid doses and concentrations (**Figure 2**). The doses tested were: 0.9% AA in NaCl solution (volume injected: 0.25mL/100 grams), 2.0% AA in NaCl solution (volume injected: 3.0mL/1000 grams), and 0.9% AA in NaCl solution (volume injected: 1.0mL/100 grams). After several dosing studies, we confirmed that the optimal Acetic Acid dose was our low dose, 0.9% AA in NaCl solution (volume injected: 0.25mL/100 grams). This dose yields similar results to those collected at the Feinstein Institute and seen in the literature (animals display writhing behavior for approximately 40% of observation periods). See **Figure 2** for the AA dose response data. In addition, we confirmed the time course of Acetic Acid-induced writhing behavior to determine the optimal time for imaging and morphine administration.

Second, the effects of two morphine doses described in the original project were examined on AA-stimulated writhing.

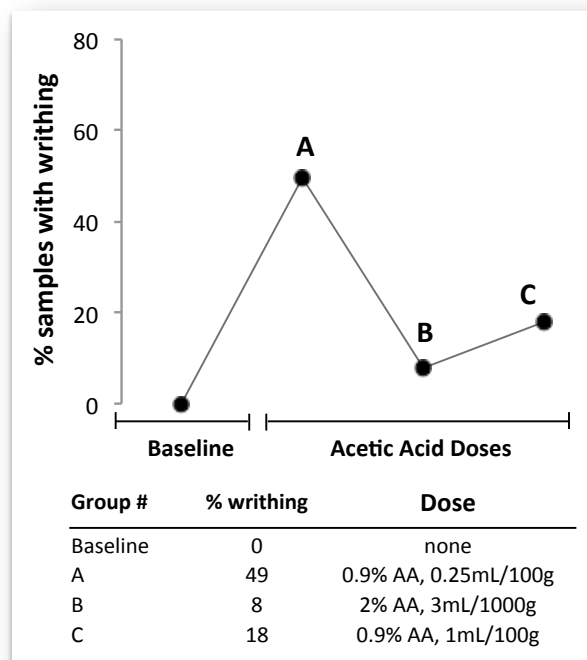


Figure 2. Acetic Acid Dose Response. Dose effects of acid-stimulated writhing. Based on this data, our optimal dose of AA was established at 0.9% AA given in 0.25 mL/100 g weight.

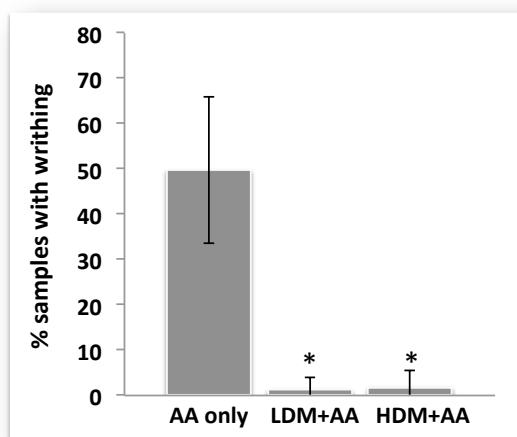


Figure 3. Morphine-induced changes in AA-induced writhing behaviors. LDM = Low Dose Morphine (5 mg/kg), HDM = High Dose Morphine (15 mg/kg). Morphine was administered 10 minutes prior to AA. Animals were monitored for 60 minutes and yielded a significant difference at the $p < 0.05$ level compared to AA only.

Morphine (5 mg/kg or 15 mg/kg) was administered 10 minutes prior to AA, and behavior was observed for 60-minutes following AA injection. The attenuation of AA induced writhing by both doses of morphine is clear in **Figure 3**.

3. Combined AA and morphine in the context of Conditioned Place Aversion (CPA) and reward (CPP)

In these studies, we first determined the dose of AA that produced a CPA (avoiding the chamber where AA was administered) and where the morphine treatment blocked this CPA and became rewarding despite prior treatment with AA. These most recent studies demonstrated that 15 mg/kg morphine given to animals in which a CPA was produced, was sufficient to reverse the effects of conditioning with AA, producing a CPP instead.

Key Research Accomplishments

Since this project has resumed, we have achieved the following accomplishments:

- Established the dose at which AA produces a CPA
- Shown that morphine, at high doses, can block this CPA and is rewarding
- Shown that the initial preference of an animal subject is not predictive of its eventual preference; this will make the rest of the project much more efficient
- Demonstrated that the place aversion as well as the writhing behavior can be dose-dependently blocked with supra-analgesic doses of morphine
- Streamlined computerized analysis of AA-induced place aversion and the effect of morphine to block this effect, removing subjective interpretations of animal behavior. This includes simultaneous analysis of conditioned aversion, writhing, locomotion, and conditioned reward with high-dose morphine.

Reportable Outcomes

Manuscripts, Abstracts and Presentations

1. Talan A, Steuer E, Colonna E, Mathson K, Hlynialuk C, Schiffer WK. Predictive validity of conditioned pretest in place conditioning paradigms. Life Sciences (in preparation)
2. Schiffer WK, Talan A, Steuer, E et al. Cue Induced changes in dopamine are of the same magnitude as cocaine-induced changes in dopamine. Neuropsychopharmacology (in preparation)

3. Schiffer WK, Talan A, Carrion J, Steuer E, Mathson K, Hlynialuk C. Positron Emission Tomography measures common patterns of metabolic brain activation in awake animals during the Formalin Paw Test and the Acetic Acid Writhing Test. Pain (in preparation)
4. Schiffer WK, Brodie JD, Carrion J, Talan A. [^{11}C]-Raclopride measures of the dopaminergic response to acute pain in freely moving animals: Correlation with behavioral observations. European Journal of Pain (in revision)

Conclusion

At a time when our soldiers are especially vulnerable to mental illness and substance abuse, we offer a potential approach to decrease the widespread risk of opiate addiction in this population. Preclinical imaging studies aim to build a foundation for effective clinical drug development, and the results from the final phase of this study will be significant on multiple levels in our understanding of pain, opiate administration and addiction.

We have made substantive progress in establishing an effective protocol to model this risk, and the further analysis of preclinical imaging data described for the last months of the proposal will reveal more about how morphine affects AA-induced pain, at what point it becomes rewarding, and how GVG affects this reward. In this, we have shown that combined with place conditioning, the measurable effects of AA-induced aversion can be reversed with morphine treatment to become measurable effects of morphine-induced reward in the presence of AA treatment.

By providing this data on opioid and CPP behavior in a pain context, we are nevertheless opening an important research avenue with translational potential and numerous questions to address concerning chronic opioid treatments, potential vulnerability factors such as individual susceptibility, the type of chronic pain. From a neuroscience perspective, this project also raises the question of the underlying mechanism leading to the altered opioid response of reward systems in the context of chronic pain; a question that can be answered directly using our imaging strategy in freely moving animals.

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